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Analytical Methods Section

Detection of common-wheat (*Triticum aestivum*) flour in Durum-wheat (*Triticum durum*) semolina by reverse-phase high-performance liquid chromatography (RP-HPLC) of specific albumins

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A method for detecting common-wheat flour in Durum-wheat semolina and low-heat dried pasta by means of RP-HPLC of water-soluble protein is described. This method provides separation of the same specific protein fractions as those determined by IEF according to the Italian Official Method (*Official Italian Journal*, No. 4, 5 January 1980). A faster sample preparation on solid-phase extraction (SPE) of protein can be adopted. This RP-HPLC method proves to be repeatable (coefficient of variation less than 4%), to have high sensitivity (less than 1% of common-wheat flour detectable), and an accuracy comparable to that of the IEF method. The RP-HPLC method cannot be applied to protein extracted from high-heat dried pasta. The HPLC patterns obtained are poorly resolved and a correct quantification of common-wheat flour cannot be achieved.

INTRODUCTION

Italian law (OIJ, 1967) lays down rules for the manufacture and marketing of pasta which must be prepared only with Durum-wheat (*Triticum durum*) semolina. National legislation of other Member States of the European Community does not allow pasta of common wheat (*Triticum aestivum*) or of mixtures of common and Durum wheat to be marketable. Therefore the analytical determination of common-wheat flour in Durum-wheat semolina is important in the protection of pasta products.

Several protein components were studied in order to distinguish the two species of wheat. Indeed protein, because it carries genetic information, can be assumed to be a 'genetic marker' of the wheat. The electrophoretic methods proposed by Kobrehel *et al.* (1985) involve the extraction of alcohol-soluble protein and the detection of low-mobility 1-D omega-gliadins. Nevertheless the evaluation of slow-moving omega-gliadins could lead to errors and misinterpretations in evaluating common wheat because its cultivars do not contain the same amount of 1-D omega-gliadins (absent in some cultivars). Alternatively, water-soluble protein fractions coded by the D genome have been widely used. Feillet and Kobrehel (1975) investigated water-soluble protein by an electrophoretic method demonstrating the presence of a specific polyphenol oxidase. In the method of Resmini (1968), some specific protein fractions, named 'A', 'B', 'C' in the case of common wheat and '1', '2', '3' in the case of Durum wheat, were extracted with 0·8 M $(NH_4)_2SO_4$ and 0·1 M $MgSO_4$ solution, salted out with 1·2 M $(NH_4)_2SO_4$ and separated by polyacrylamide gel electrophoresis. Isoelectrofocusing (IEF) was successfully adopted (Resmini & De Bernbardi, 1976) and this last method is now the official one in Italy for detecting common-wheat flour in semolina and pasta (OIJ, 1980). More recently Ainsworth *et al.* (1984) separated water-soluble protein by IEF with a view to distinguishing Durum wheat from common wheat on the basis of particular esterase fractions. Concerning the biochemical characterization of these markers, it is likely that most of them are alpha-amylase inhibitors, with the exception of poly-

phenoloxidase and esterase (Garcia Olmedo *et al.*, 1987).

HPLC is becoming a widely applied analytical technique for studying cereal protein. HPLC of gluten protein essentially brought varietal identification and prediction of manufacturing quality (Dachkevitch & Autran, 1989). Only recently McCarthy *et al.* (1990) proposed detection of common-wheat flour in semolina by RP-HPLC of gliadins. Low-pressure liquid chromatography (LPLC) of albumins and globulins allowed several cereal proteins to be biochemically characterized (Marchylo & Kruger, 1987). On the other hand, RP-HPLC of albumins was not adopted until now for detecting common-wheat flour in Durum-wheat products.

The aim of this paper was to apply RP-HPLC in the quantification of the same specific albumin fractions as those provided by the Italian Official Method.

MATERIALS AND METHODS

Five samples of pure Durum-wheat semolina (purity checked by IEF), nine commercial samples of Durum-wheat semolina and five of common-wheat flour were randomly collected on the Italian market.

Standard samples were prepared by mixing pure Durum-wheat semolina with different amounts of common-wheat flour (0·5, 1·0, 2·0, 3·0, 4·0 and 7·0%).

Two pasta samples, containing known amounts of common-wheat flour, were produced and dried either at low temperature (60°C for 20 h) or at medium temperature (92°C for 40 min and 78°C for 4 h) at industrial cycle conditions.

Sample preparation

LPLC

One kilogram of flour or semolina was submitted to protein extraction according to Resmini (1968); the extracted material was dissolved in 50 ml of water, dialyzed against distilled water and freeze-dried.

RP-HPLC

Procedure (a). Ten grams of flour, semolina or ground pasta were extracted as indicated for LPLC and the material obtained dissolved in 300 μl 3 M urea and filtered (0·45 μm Millipore, Bedford, MA); 20 μl of filtrate were submitted to RP-HPLC investigation.

Procedure (b). Ten grams of flour, semolina or ground pasta were extracted for 30 min at room temperature with 35 ml 0·8 M ammonium sulphate and 0·1 M magnesium sulphate solution adjusted to pH 3·25; the solution was centrifuged at 3000 *g* for 10 min; 0·5 ml supernatant was loaded in a prewetted Sepak C_{18} cartridge (Millipore) and eluted with 0·5 ml aqueous methanol 15% and 1 ml aqueous acetonitrile 30%; the last 1 ml eluted was submitted to RP-HPLC.

Chromatographic determination

Instrumentation

The LPLC system was a Model P-1 pump (LKB, Uppsala, Sweden) with a UV detector Model Uvicord SII (LKB). A S-Sepharose Fast Flow column (20 mm i.d. × 300 mm, 10 μm particle size, LKB) was used. The HPLC system was a Model 625 LC multisolvent delivery system (Waters, Milford, MA), with an injection system Model 465 (Kontron Instruments, Milan, Italy) and a UV detector Model 486 (Waters). A PLRP-S column (4·6 mm i.d. × 150 mm, PSDB, 8 μm particle size, Polymer Lab, Shropshire, UK) was used.

Separation conditions

LPLC. Preparative separation of common-wheat protein was run isocratically with 0·025 M sodium acetate buffer, pH 6·0; the same buffer and a linear 360 min elution gradient from 0 to 30 mM NaCl was used for Durum-wheat protein. Column temperature, 20°C; flow rate, 1 ml min^{-1}; UV detection, 280 nm, 1 AUFS. The eluted fractions corresponding to the interesting peaks were collected every 2 min, dialyzed, freeze-dried and submitted to IEF investigation.

RP-HPLC. A linear elution gradient of water (solvent A) and acetonitrile (solvent B), both containing 0·1% (v/v) trifluoracetic acid (TFA), was used. Elution gradient as solvent B proportion: 0–15 min, 27–31%; 15–17 min, 31–100%; 17–19 min, 100%; 21–30 min, 27%. Column temperature, 50°C; flow rate, 1 ml min^{-1}; UV detection, 210 nm, 0·1 AUFS. Specific albumins were quantified by a calibration curve where the ratio of the peaks 'A'/'1' (calculated as peak-height values), was plotted versus the percentage of common-wheat flour in semolina. The highest peaks were measured using a workstation Maxima 820 (Waters, Milford, MA). The eluted fractions corresponding to the interesting peaks were collected and concentrated to 100 μl under vacuum.

Electrophoretic determination

IEF (pH 3·5–9·5) of the extracted protein was according to the Italian Official Method (OIJ, 1980). The quantitative evaluation of the gels was performed by both visual examination and densitometric scanning (Camag Scanner II, Muttenz, Switzerland) at 630 nm.

Total protein

The protein content of flour, semolina or pasta was determined by AACC method (1983).

RESULTS AND DISCUSSION

Under the conditions adopted for RP-HPLC, the albumin fractions of common and Durum wheat extracted according to procedure (a) are well separated

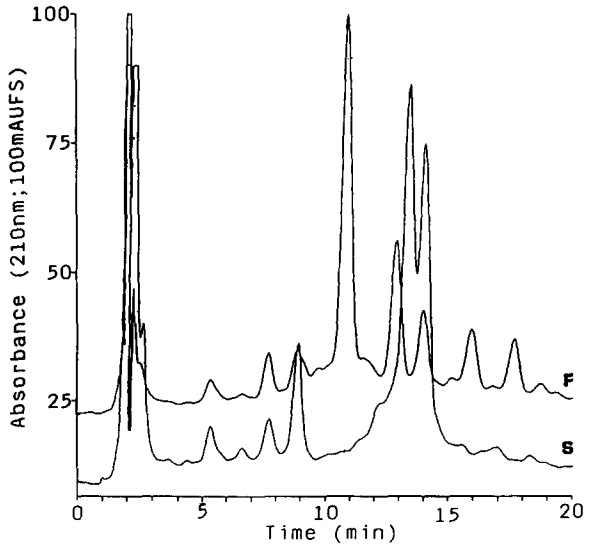
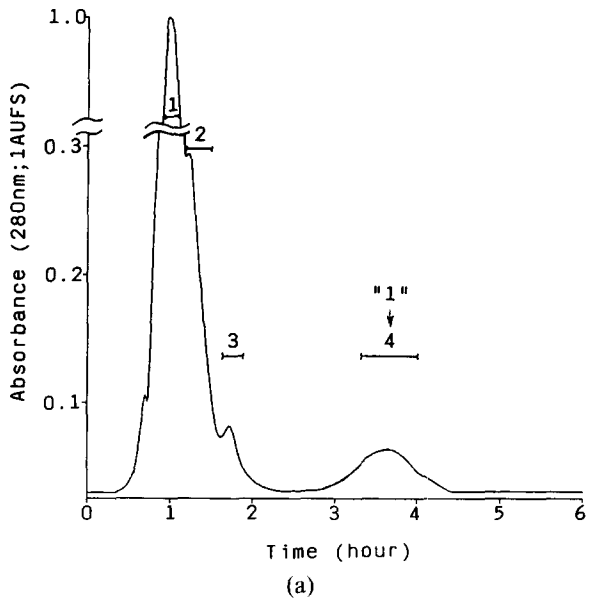
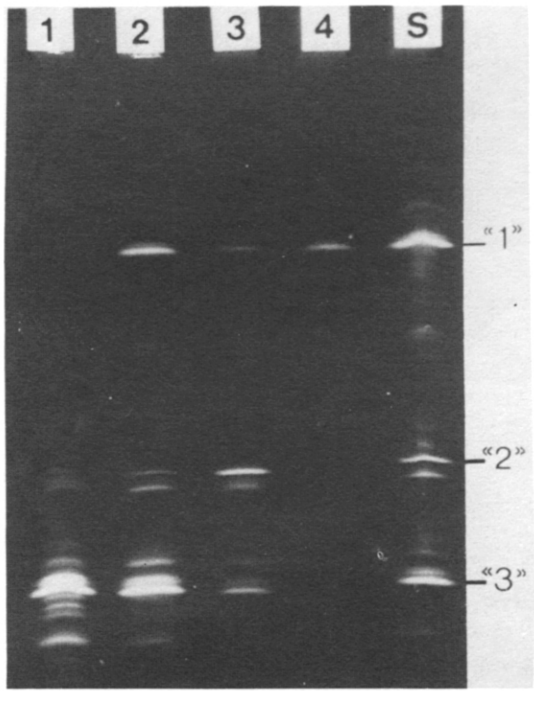


Fig. 1. RP-HPLC of albumins of common-wheat flour (F) and Durum-wheat semolina (S).



(a)



(b)

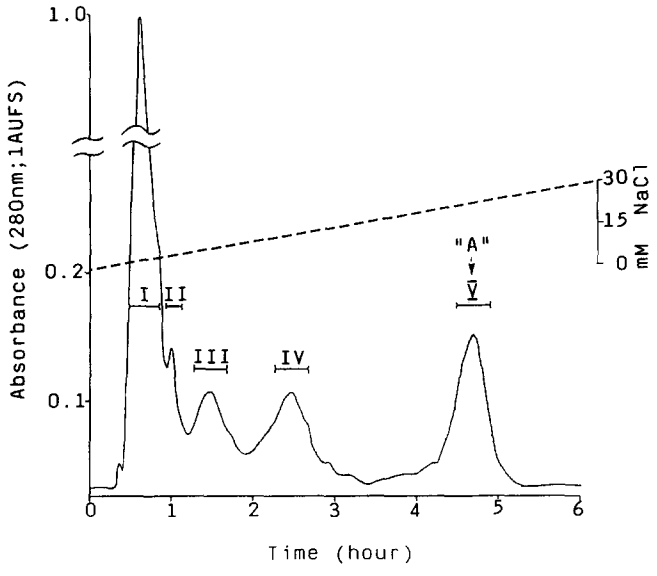
Fig. 2. (a) LPLC of albumins of Durum wheat semolina; I—I, collected fractions. (b) IEF of collected fractions; S, Durum-wheat semolina.

and differential patterns were obtained (Fig. 1). Moreover, similar patterns were obtained for samples of common-wheat flour and Durum-wheat semolina of different origin.

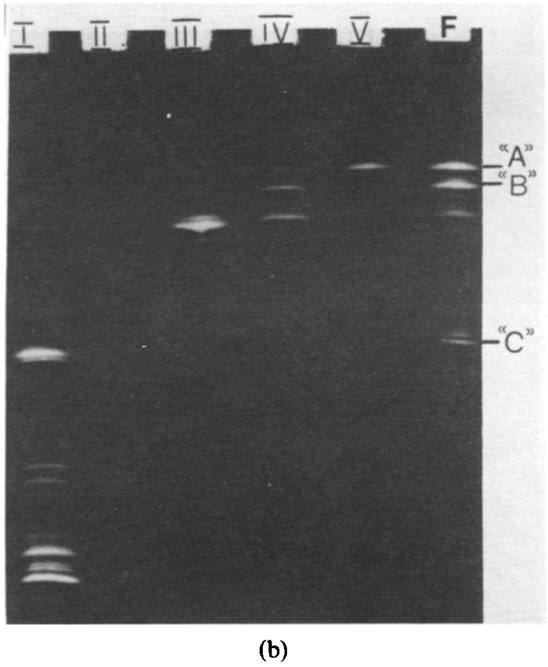
On this basis, an attempt was made to establish whether a correspondence between chromatographic peaks and IEF bands could exist. Direct identification of the chromatographic peaks by means of IEF was not possible, because of the interference of TFA on the formation of the pH gradient, thereby affecting separation of the bands as well. Thus extracted protein was separated by LPLC (Figs 2(a) and 3(a)) and the eluted fractions were submitted to IEF. The fractions 4 and $\bar{V}$ correspond to the IEF bands '1' and 'A' respectively (Figs 2(b) and 3(b)). RP-HPLC of the LPLC fractions allows the chromatographic peaks corresponding to the above-mentioned electrophoretic bands to be identified (Fig. 4).

RP-HPLC patterns of the standard mixtures of common with Durum wheat are reported in Fig. 5. The relationship existing between common-wheat flour amount and the ratio of peaks 'A'/'1' (calculated as peak-height values), is linear in the range 0–7%. The calibration curve obtained with seven samples (three replications each) is highly significant (r^2 = 0·98) and the highest residual found between true and analytical data is equal to 0·6 (mean = 0·3). Similar results were obtained for the mixtures of common-wheat flours of different origins with the same Durum-wheat semolina. Seven replications on three different days of the same sample mixture containing 3·0% of common-wheat flour gave an average value of 3·1% and CV of 3·24.

In order to evaluate the reliability of the RP-HPLC method, five samples of pure (purity checked by IEF) semolina with different protein content (ranging from 11·0 up to 13·1%) were mixed with different amounts of the same flour and the related calibration curves were obtained. The same standard sample (in the range 0–4%) was quantified and the highest residual of 1% unit was found when two different curves were used. This error is comparable to that of IEF, while the quantification of common-wheat flour appears to be independent of the semolina used. On this basis, nine commercial semolina samples were analyzed on RP-HPLC and IEF, using the same standard samples. Both methods gave the same quantitative data proving that RP-HPLC and densitometric scanning of the electrophoretic gels have a comparable precision. The quantification of low levels (<1%) of common-wheat flour performed by visual examination of gels is more sensitive than by densitometric scanning. The RP-HPLC method allows small amounts (<3%) of common-wheat flour generally reported as 'agronomical impurities' of Durum wheat (OJ, 1992) to be accurately quantified.



(a)



(b)

Fig. 3. (a) LPLC of albumins of common-wheat flour; I—I, collected fractions. (b\) IEF of collected fractions; F, common-wheat flour.

The sample-extraction procedure (b) involves the use of simple instrumentation and a super-centrifuge is not necessary. Under these extraction conditions, as well, the calibration curve obtained by RP-HPLC is highly significant (r^2 = 0·98) and the highest residual found between true and analytical data is equal to 0·7.

The RP-HPLC method was successfully applied for separating the protein extracted from low-heat dried pasta according to procedure (a). RP-HPLC of pasta dried at high temperature does not produce well-resolved patterns which are not comparable with those of semolina samples. This problem was not observed in IEF patterns where important changes can only occur when pasta is dried at very high temperatures (Resmini *et al.*, 1975). Generally, pasta drying promotes a partial insolubilization of water-soluble proteins. Actually, it is not clear whether

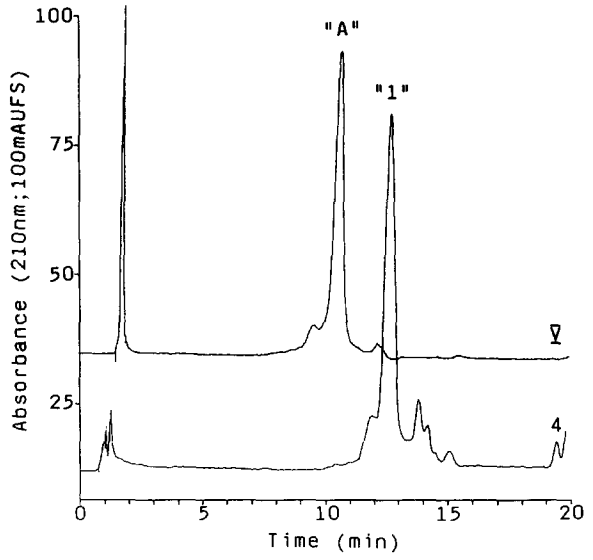


Fig. 4. RP-HPLC of fractions 4 and $\bar{V}$ eluted from LPLC of Durum-wheat semolina and common-wheat flour (see Figs 2 and 3).

this insolubilization is due to formation of covalent bonds between soluble protein and insoluble components of semolina or to reciprocal aggregation of albumins and subsequent coagulation (Dexter & Matsuo, 1977). Probably the thermal treatment causes unfolding of the native coiled structure, involving changes of the surface hydrophobicity. These changes can negatively affect the separation of peaks under RP-HPLC. When medium- or high-heat dried pasta must be analyzed, the quantification of common-wheat flour by means of RP-HPLC is strictly related both to a sufficient resolution of chromatographic pattern and to the availability of samples of standard pasta processed under the same thermal conditions as those of the sample analyzed.

Data from this work prove that RP-HPLC of specific albumins is a very sensitive and accurate method for detecting common-wheat flour in Durum-

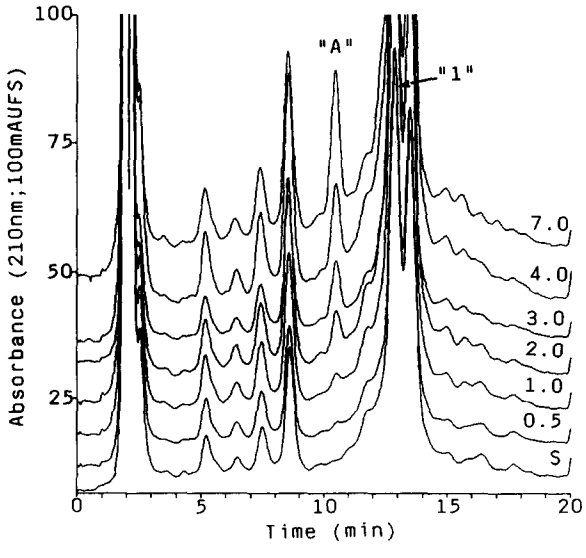


Fig. 5. RP-HPLC patterns of Durum-wheat semolina (S) containing different amounts of common-wheat flour (0·5, 1·0, 2·0, 3·0, 4·0 and 7·0% w/w).

wheat semolina. In this method, common-wheat flour is determined by evaluating the same specific protein fractions as those provided by the Italian Official Method, and the quantitative data obtained with the two methods are comparable. The same accuracy is achieved when sample preparation by SPE is used.

Owing to the several advantages of HPLC, such as higher resolving power, sensitivity and automatic sample handling, the method here described can be easily adopted in customs and industry laboratories which find the electrophoretic techniques difficult to apply.

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